OBSERVATIONS ON THE STAINING OF ENCAPSULATED BACTERIA WITH PARTICULAR REFERENCE TO PNEUMOCOCCI AND STREPTOCOCCI.*

LEO BUERGER.

(From the Pathological Laboratory, Mt. Sinai Hospital, New York.)

IN the past few years quite a number of contributions to methods of staining encapsulated microorganisms have appeared in the literature. Yet there exists no lack of disagreement amongst the workers in this field both as regards the advantages of the various methods that have been proposed and as to the morphology of pneumococci and streptococci. It is the purpose of this paper to help clear up some of the fallacious ideas that have been put forth, and to give the results of experiences gathered in the laboratory of Mt. Sinai Hospital during a period of about three years.

The procedure for capsule staining that has given us the most satisfactory results appeared in a previous publication[1] where it was thus described.

The necessary solutions are as follows: (1) Müller's fluid (bichromate of potassium 2, 5 grms.; sulphate of sodium, 1.0 grm.; water, 100 c.c.) saturated with bichloride of mercury (ordinarily about 5 per cent). (2) Beef, human, or other blood serum diluted with an equal amount of normal salt solution; or ascitic or pleural fluid. (3) 80–95 per cent alcohol. (4) Tincture of iodine, U. S. P. (5) Freshly prepared stain;[2] anilin water gentian violet, made up as follows: anilin oil 10; water 100; shake, filter, and add 5 c.c. saturated alcoholic solution of gentian violet. (6) Two per cent watery salt solution.

The culture is thinly and carefully spread on a perfectly clean cover-slip by means of a drop of diluted serum. Just as the edges begin to dry[3] the fixing fluid, solution No. 1, is poured on, the cover gently warmed over the flame for about three seconds, rapidly washed in water, flushed once with alcohol, and then treated with iodine for one to two minutes. The iodine is in turn thoroughly washed off with alcohol, and the specimen dried in the air. Staining for two seconds, and washing with salt solution completes the procedure. The specimen is mounted in the salt solution and ringed with vaselin.

In order to attain uniform results—and this in our experience holds true with other methods of staining—a serous fluid of the

* Received for publication March 28, 1907.

[1] *Med. News*, 1904, 85, p. 1117; also *Centralbl. f. Bakt.*, 1905, Orig. 39, pp. 216, 335.

[2] A quantity sufficient to last for about four days ought to be prepared.

[3] Good results may even be obtained after the cover-slip preparation has been allowed to dry in the air; however, the method described is preferable because it gives much clearer fields.

proper quality must be selected. It has been our custom to employ sterile ascitic fluid which has been tested on typical pneumococci and to preserve some of it for capsule staining. A small quantity (25 c.c.) will suffice for a year's work.

Since the serous fluid has for its object the preservation of the capsules, the culture must be well impregnated with the drop on the slide before spreading. This is especially important in the preparation of stains of streptococci. A tiny bit of the culture, therefore, must be gently mixed with the drop of serum and then spread, taking care not to drag the organisms beyond the limits of the fluid. This mixing process is important when we have to deal with certain mucoid bacilli and *Strept. mucosus capsulatus*. Here, when the growths are very viscid, it is advantageous to make an emulsion in a few drops of serum on a separate slide, and then to transfer some of it to the cover-slip.

The fixing fluid should not remain on the cover-slip longer than three to five seconds; at about this time the specimen becomes opaque, an appearance that indicates the completion of the fixing process.

It is essential, furthermore, to make a practice of examining for capsules only on the most favorable media. These are the glucose-serum agar and a well-prepared Loeffler's coagulated serum. In our routine work we rely upon a 0.5–2 per cent glucose-serum agar made up with ascitic fluid, the latter constituting about one-fourth of the volume of the medium.

If we employ this method on a large number of pneumococci, streptococci, and encapsulated bacilli, we meet with certain regularly defined types of capsules. One of these is characteristic for the pneumococcus, another for certain streptococci, and still another for mucoid streptococci and mucoid bacilli—and so we may distinguish the following types:

1. The pneumococcus type.
2. The streptococcus type.
3. The mucoid type.

The pneumococcus type.—This type which is found on the most favorable culture media is illustrated by Fig. 1.[1] Here the diplococcus

[1] I am indebted to Dr. F. S. Mandlebaum, Director of the Laboratory, for the microphotographs reproduced in these pages.

is surrounded by a deeply staining, sharply defined, elliptical band, situated at some distance from the body of the bacterium and separated from it by a clear area. This clear interspace is sometimes stained but usually less deeply than the limiting membrane. The capsules have an elliptical outline and when they surround chains show distinct constrictions between the diplococci.

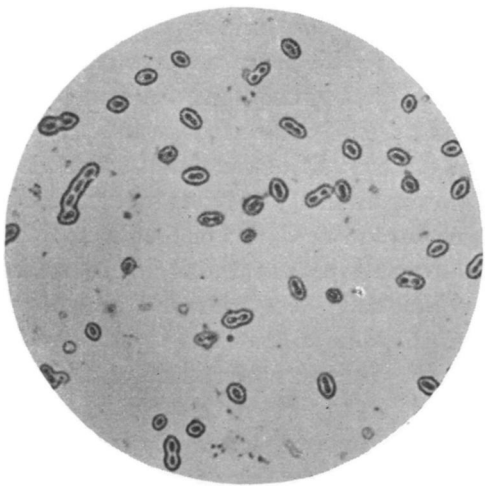

FIG. 1.—Typical pneumococcus.

When the conditions for growth are unfavorable, or after a number of transplantations, or at times in the very first cultures from the blood of the patients who have harbored pneumococcus for a considerable period of time, these typical appearances may be absent. The capsule degenerates, becomes narrower, and assumes the type characteristic for certain streptococci, or may even be entirely lost (see Fig. 2.). The strains vary considerably as to the length of time that the typical or well-matured form is retained. By subinoculations on very favorable media the usual form may often be restored. This also may be effected by inoculation into susceptible animals (mouse and rabbit).

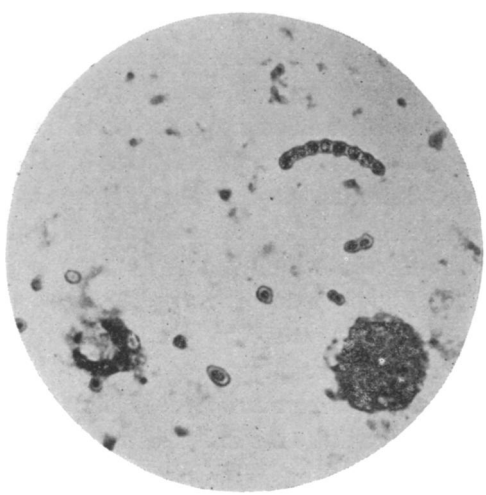

FIG. 2.—Degenerate pneumococci in an old empyema exudate.

In a study of a large number of strains of pneumococci, it was found that whenever the typical forms were present, the organism presented the biological and cultural features peculiar to the pneumococcus. Such organisms fermented inulin, although with some strains this did not occur regularly in every generation. Thus we consider the morphological characteristics of considerable importance in diagnosis.

The streptococcus type.—If we stain a large number of strains of pathogenic streptococci we note that there are some forms which show no capsule under any conditions, others which possess narrow capsules, and finally those that belong to the group *Strept. mucosus capsulatus*. The streptococcus type is shown in Fig. 3, an organism isolated by blood-culture from a patient suffering from a rather chronic form of streptococcemia. The capsule is narrow, the periphery rather close to the body of the organism (usually even more closely adapted than in the figure), and frequently shows constrictions separating either the single elements or the pairs of hemispherical elements when such are present. Pneumococci, when they degenerate both in culture or in old exudates, frequently have such capsules. They cannot then be differentiated morphologically from streptococci.

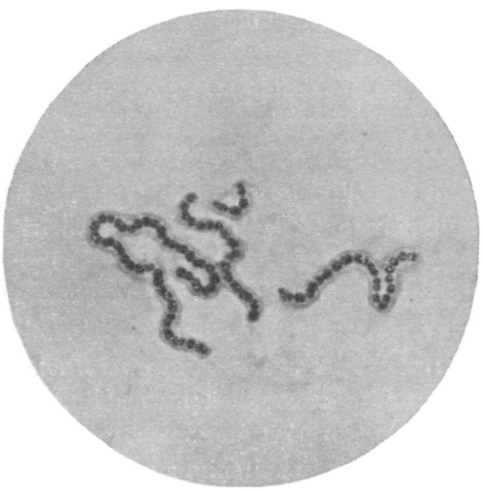

FIG. 3.—Streptococcus with the streptococcus type of capsule.

Fully 25 per cent of the pyogenic streptococci received in the laboratory in the course of routine bacteriological work show capsules. With a little experience it is not a difficult matter to predict whether a streptococcus will show a capsule or not, by observing the character of the growth on glucose-serum agar. Many streptococci, such as

those usually present in the feces, in the milk, and also some pathogenic varieties, show dry, often whitish, colonies. These are not encapsulated. Others, however, show moist watery colonies, and still others have even a mucoid appearance, particularly in the first generations. Such organisms regularly have the streptococcus type of capsule.

In a paper on *Strept. mucosus capsulatus*[1] I drew attention to the importance of differentiating some of these streptococci from *Strept. mucosus*, as follows:[2]

> There are streptococci which have a rich watery growth on serum agar and glucose serum agar, but that do not belong to the group *Strept. mucosus capsulatus*. These were found in the blood of cases of "ulcerative endocarditis," as well as in various local infectious processes. The colonies resemble those of the *Strept. mucosus* very much. When stained with the author's method they show the narrow "streptococcus type" of capsule. On glucose-serum agar they cause precipitation whereas the *Strept. mucosus* does not. Twenty-four hours after isolation from the blood of a patient, the colonies on the plates may be like those of *Strept. mucosus*. Later however, they cause distinct hemolysis, whereas the green color and absence of hemolysis are characteristic for *Strept. mucosus*. Such streptococci do not form a mucoid exudate when inoculated into animals, nor do they show the typical morphology of *Strept. mucosus* in the tissues or in the blood. They usually lose their watery appearance after a few transplantations.

None of the strains that I have examined fermented inulin.

The mucoid type.—Strept. mucosus capsulatus possesses a large capsule that stains diffusely and whose periphery has a tendency to be irregular and less well defined than that of the pneumococcus. It surrounds the diplococci or chains usually without showing any constrictions between the elements (see Figs. 4 and 5). Similar capsules are to be seen on certain strains of the Friedlander bacillus and of *M. tetragenus*.

Wadsworth[3] in a recent publication gives a critical review of some of the methods of staining encapsulated organisms and suggests a new method in which formalin is employed as a fixative. A similar method was tried by me several years ago.[4] I stated then that formalin was less reliable than the Müller-bichloride fixation. To quote, "Auf andere Fixationsmittel, wie Formalin und Flemingsche Flüssigkeit,

[1] Buerger, *Centralbl. f. Bakt.*, 1906, 41, p. 511.

[2] Translation.

[3] *Jour. Infect. Dis.*, 1906, 3, p. 610.

[4] *Centralbl. f. Bakt.*, 1905, Orig. 39, p. 219.

habe ich verzichtet, da dieselben kein klares Gesichtsfeld gaben und überdies vor der oben empfohlenen Lösung keinerlei Vorteile voraus hatten." For the staining of organisms with very well-developed capsules the method of Hiss and the formalin fixation give excellent results and are simple and reliable. However, where the capsules are poorly developed, where the degenerative forms make their appearance, and in the case of streptococci not of the *mucosus* group, the method of Hiss fails to show capsules, and the formalin method gives varying results.

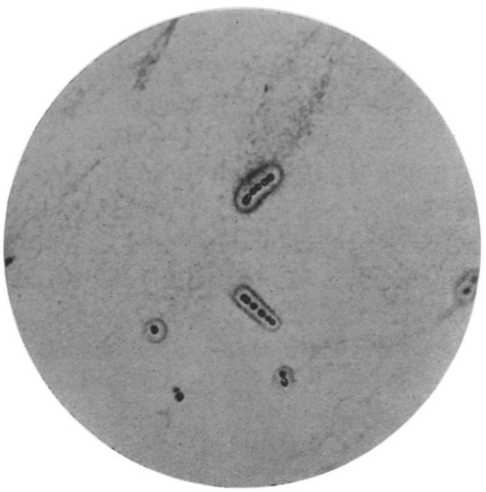

FIG. 4.—*Streptococcus mucosus capsulatus.*

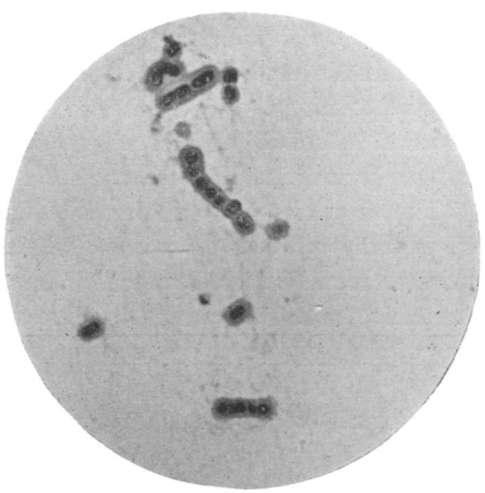

FIG. 5.—*Streptococcus mucosus capsulatus.*

In speaking of my method Wadsworth says, "Buerger fixes the smear of encapsulated pneumococci in Müller's fluid saturated with bichloride for one half-minute, and washes in water." In the articles in which the method is described and which he quotes, it is distinctly stated that the fixative is to remain on the coverslip for *three seconds* over the flame. The uniformly good results obtained in our laboratory by following out these directions make the footnote which Wadsworth adds—"In my experience

with this method longer exposures are advisable; in fact, essential" —very difficult to understand.

As regards the combined Gram and capsule stains of organisms in culture, Wadsworth is in error when he says, "With these methods of Smith and Buerger the bacterial cells, under favorable conditions, may be stained by the Gram stain and demonstrated with capsules." Smith's stain was only used in the staining of sputum or exudates. With the combined method, as first proposed by me, we have never failed to demonstrate a Gram negative capsule and a Gram positive cell body on pneumococci that showed capsules by the simple stain.[1]

Earlier, in this paper, I dwelt upon the question of capsules on streptococci, because this matter seems to be still a mooted one. Thus Wadsworth says, "Streptococci, in my experience, have rarely given definite capsules with these formalin methods," and in a footnote, "Occasionally a faint, hazy periphery, suggesting a shrunken or degenerated, partially dissolved capsule, was noted." It is hard to reconcile these statements with my own results. I have been able to demonstrate capsules on quite a large number of strains of streptococci with the formalin method, but more frequently with the Müller—bichloride of mercury fixation. As I have said before fully 25 per cent of streptococci examined in the laboratory, showed the "streptococcus type" of capsule. The formalin method gave results in a large number of these, the Hiss method rarely.

When we consider Wadsworth's failure to get the best possible results with both my own and the formalin method we can see the *rationale* for his statement, "The morphological differences of the capsules of the pneumococci, as compared with other encapsulated organisms resembling the pneumoccocus, which Buerger obtained with his simple stain, and upon which he lays so much stress, depend chiefly upon the varying stages of development or degeneration and solution, and upon the degree of decolorization. They are in no sense differential." The morphological types which I have pointed out represent not, as he would have it, stages of degeneration, but the highest developmental forms of the various organism. Pneumo-

[1] The combined capsule and Gram stain is carried out as follows: Spread in serum; fix with Müller bichloride of mercury (3–5 seconds); wash with water; tincture of iodine (1–2 min.); alcohol, dry; anilin water gentian violet (5–10 per cent); drain; Gram's iodine; drain; alcohol; water; aqueous fuchsin 10 per cent (2 min.); water; mount in water.

cocci when grown under favorable conditions show the pneumococcus type. Streptococci with capsules present these only under similar conditions and may lose them in subcultures. But the streptococcus, the type with the capsule, represents the most mature form of that organism. Whoever has demonstrated it on luxuriantly growing strains, and has missed it in later generations, will recognize the correctness of this assertion. Apparently the development of the streptococcus capsule is not as high as that of the pneumococcus.

In previous communications I discussed at length the differentiation of *Strept. mucosus* from the pneumococcus. Wadsworth thinks that morphological distinctions cannot be made. It is difficult to understand this viewpoint particularly when we glance at the differences shown in Figs. 1, 4, and 5. Still in another place he says, "The methods of Guarnieri, of Welch, and of Buerger, reliable, though in one way or another complicated, all give temporary mounts, and are thus unsatisfactory." Both the Welch and my method can give permanent mounts, specimens of which I have had in my possession for more than two years. It is true that these are inferior to the fresh mounts. For our purposes of diagnosis, however, it is far more important to be able to differentiate pneumococci from streptococci aided by reliable staining methods than to add permanent specimens to our collection.

It may be justly contended that the appearances described by me as typical for various encapsulated organisms are to some extent fashioned by the material employed in staining. Thus the bichloride of mercury in the fixative may, it is true, be responsible for a certain amount of shrinkage of the capsule substance. However, artificial appearances of some kind or other are produced by all methods. The serum used for the spreads may also have some influence on the nature of the capsule. For routine work, where it is important to obtain appearances that are of value in differentiation, the method adopted by us seems to be the most satisfactory.